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Kinetic Interplay between Herpes Simplex Virus Type 1 Helicase-Primase and Polymerase

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BY

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LIST OF ABBREVIATIONS

CNS	Central nervous system
CTL	Cytotoxic lymphocyte
EBV	Epstein-Barr virus
HCMV	Human cytomegalovirus
HHV-6	Human herpesvirus type 6
HHV-7	Human herpesvirus type 7
HHV-8	Human herpesvirus type 8
HLA-1	Human leukocyte antigen complex class 1
HSE	Herpes simplex encephalitis
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
MHC-1	Major histocompatibility complex class 1
PAGE	Polyacrylamide gel electrophoresis
SEM	Skin, eye, and/or mouth disease
VSV	Varicella-zoster virus

1 INTRODUCTION

Viruses have long been used to explore the mysteries of DNA replication. Herpes simplex virus type 1 is one of the most researched viral models and has allowed us to better understand not only viral genome duplication, but also that of prokaryotes and eukaryotes. As one of the most widespread and efficient infectious agents known, herpes simplex virus type 1 provides a fascinating opportunity to elucidate the mechanisms of gene replication.

The HSV-1 genome encodes several replication proteins that form the herpes replisome. Included in these are the genes that encode herpes helicase-primase (UL5/8/52) and herpes polymerase (UL30/42). UL5/8/52 is a heterotrimeric, lagging-strand helicase-primase which uses energy from NTP hydrolysis to unwind DNA ahead of the rest of the replisome at the replication fork. UL5/8/52 and UL30/42 may act together, displaying positive cooperativity in order to efficiently unwind and replicate the herpes genome.

In this study, we further examined the replication of the HSV-1 genome, with specific emphasis on the potential consequences of mechanistic coupling between UL5/8/52 and UL30/42. The turnover rate for polymerization of dNTPs by UL30/42 is much more rapid than the rate of unwinding duplex DNA by UL5/8/52. Because HSV-1 requires formation of single-stranded DNA at the replication fork before polymerization of daughter strands, we predicted that cooperative interaction between UL30/42 and UL5/8/52 must occur in order to enhance the rate of unwinding. We found that while cooperative effects may exist in the interaction of UL5/8/52 and UL30/42, there are many other factors that affect this complex process. Additionally, we discovered that the reaction

conditions did not sustain kinetic synergy between the enzyme complexes in experiments comparing UL5/8/52 activity alone and in coordination with UL30/42. UL5/8/52 has previously been shown to be an inefficient enzyme, with estimated unwinding rates close to two nucleotides per second (9). We observed a similar characteristic inefficiency. Surprisingly, UL30/42 was not able to enhance the rate of unwinding by UL5/8/52. This outcome was unexpected, given previous models of viral replication and the kinetics of these enzyme complexes.

Ultimately, the broad goal of my research is to develop better antiviral therapies by further elucidating the nature of interactions between UL5/8/52 and UL30/42, and HSV-1 replication in general. Though UL30/42 has long been a target for drugs based on nucleoside analogues, more recent approaches focus on UL5/8/52 and possible targeting of mechanistic coupling between various subunits of the HSV-1 replisome. Though HSV-1 has been extensively studied, there is still much to learn about this virus.

2 LITERATURE REVIEW

2.1 HERPESVIRIDAE

The *Herpesviridae* comprise a large family of over 120 known members, including several serious human pathogens (2). Within the herpesvirus family are three subfamilies, α, β, and γ. In turn, these subfamilies are composed of several herpesviruses that are specific to human hosts. Included among these are herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) which cause oral and genital sores; human cytomegalovirus (HCMV); human herpesviruses 6 (HHV-6) and 7 (HHV-7); human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma virus; varicella-zoster virus (VSV), cause of chickenpox and shingles; and Epstein-Barr virus (EBV), infection by which can result in mononucleosis and Burkitt's lymphoma (3). Once a host is infected by a herpesvirus, the virus will be present in the host for the rest of his or her life. The virus primarily exists in a latent state in immunocompetent individuals, and therefore rarely contributes to morbidity or mortality of these individuals. However these viruses may become reactivated in immunocompromised hosts and contribute to devastating pathogenesis.

This thesis is focused primarily on HSV-1, a member of the α-herpesvirus subfamily. It is the most extensively studied herpesvirus, but there is still much that remains to be understood about the mechanisms that contribute to HSV-1 infection in humans. Like other members of the *Herpesviridae* family, HSV-1 can exist in a lytic state in which it

actively replicates as part of a primary infection, triggering the host immune system to respond by sending cytotoxic lymphocytes (CTLs) to target the infection and induce apoptosis in infected cells. Primary infection generally occurs in the mucosal epithelium of the mouth. The characteristic cold sores indicative of a possible HSV-1 infection result from killing of the infected epithelial cells by host CTLs as well as lysis induced by the virus itself. The host is also most likely to be able to pass the virus on to other susceptible individuals while experiencing active infection (5).

After a short reproductive phase, HSV-1 is able to establish a long-term latent state in the sensory ganglia, often specifically targeting the trigeminal and olfactory nerves (6). Neurons are a particularly attractive site for latent infection by HSV-1, as well as for certain other herpesviruses, because they display very low levels of class I major histocompatibility complex (MHC-I, also known as human leukocyte antigen complex class I or HLA-I). Reduced production of MHC-I implies decreased likelihood of presentation by MHC-I of HSV-I-produced peptides, resulting in lower potential of interaction with and detection by CTLs, thus allowing HSV-1 infection to persist. During the latent stage, replication and production of viral peptides is significantly reduced, aiding in this process as well. Reactivation of HSV-1 can result in its migration from the neurons back to mucosal epithelium and recurrence of lytic infection in a manner that is not well understood (5). This highly effective virus represents an attractive model for furthering our understanding of DNA replication and the features that contribute to accurate and efficient duplication of DNA genomes.

2.2 Biological Effects of HSV-1 Infection and Current Treatment Strategies

By some estimates, over 90% of adults worldwide have a positive HSV-1 antibody titer, even though many people have never experienced symptoms of infection. Acquisition of herpesviruses is correlated with low socioeconomic status of the host, especially in cases where the virus is contracted early in life (6). There is no cure for HSV-1 infection. However, the cold sores which are a hallmark of HSV-1 infection are not generally life-threatening, though they may be irritating and painful, and generally self-resolve within two weeks (7). Those individuals who are immunocompromised due to AIDS, immunosuppressive chemotherapeutic drugs or other cancer treatments, eczema, etc., are at the greatest risk for complications.

In addition to relatively benign cold sores, HSV-1, and nearly all other members of the *Herpesviridae* family are able to cause severe central nervous system (CNS) symptoms. Herpes simplex encephalitis (HSE), known as neonatal HSV CNS disease in neonates, is the cause of significant morbidity and mortality even when treated with antiviral agents. It is believed that approximately 1 in 250,000 to 500,000 individuals per year experience HSE, which is considered the single most common cause of spontaneous, fatal encephalitis. Untreated HSE is associated with a mortality rate of 70% and a neurologic morbidity rate of nearly 97% (6). The effects of this disease are clearly non-trivial. The majority of those affected are either between 6 months and 20 years of age, or over the age of 50 years. 70% of all cases are caused by reactivated virus, and nearly all HSE cases are the specific result of HSV-1 infection (6).

Neonatal HSV CNS disease acquired peripartum has been placed in three distinct categories. These are 1) skin, eye, and/or mouth (SEM) disease which does not involve the CNS, 2) CNS disease, and 3) disseminated disease which may involve the CNS in addition to other organ systems (6). Disseminated disease in infants born in the pre-antiviral era results in a mortality rate of 85% in the first year of life with 50% suffering from permanent neurodevelopmental deficits, while those who acquired CNS disease have a 50% mortality rate in their first year of life and 66% suffered from neurodevelopmental deficits (6).

Development of drug therapies to HSV types 1 and 2 progressed significantly through the 1980s. Nucleoside analogues are the preferred approach to drug treatments, which halt DNA replication by competitively inhibiting the HSV-1 replisome and causing chain termination. Among those compounds tested for efficacy in inhibiting HSV-1 replication are acyclovir triphosphate ([9-(2-hydroxyethoxymethyl)guanosine 5'-triphosphate]), araATP (9-β-D-arabinofuranosyladenosine 5'-triphosphate), aphidicolin, (E)-5-(2-bromovinyl)-2'-deoxyuridine 5'-triphosphate, phosphonoacetate, phosphonoformate, and oxalate (3). Drugs based on nucleoside analogues commonly used today include deoxyguanosine analogues acyclovir (Zovirax), famciclovir (Famvir), penciclovir (Denavir) and valacyclovir (Valtrex) (7, 8).

Intravenous vidarabine, an adenosine analogue, and intravenous acyclovir were introduced in the early antiviral era for treatment of neonatal HSV infection. These therapies resulted in improvement of one year mortality from disseminated and CNS disease to 50% and 14% respectively, on vidarabine, and 61% and 14% respectively, on acyclovir (6). By doubling the dose of intravenous acyclovir (60mg/kg/day), these

percentages were further improved, with one year mortality from disseminated and CNS disease falling to 29% and 4% respectively (6). Acyclovir is currently the standard of care for treatment of HSV-1 infection and HSE. There are, however, further improvements that can be made on the methods for treating herpesvirus infections. Targeting of other requisite replication enzymes such as the HSV-1 helicase-primase complex may prove to be fruitful and indeed active research is occurring in this arena. By better understanding the manner in which herpesviruses replicate, we may ultimately be able to develop more effective approaches to targeting weaknesses in these processes.

2.3 Herpes Simplex Virus Type 1 Replication Overview

The HSV-1 genome is an approximately 153-kilobasepair linear DNA duplex. It contains Unique Long (U_L) and Unique Short (U_S) regions that comprise 82% and 18% of the genome, respectively. Internal repeat sequences separate the U_L and U_S sequences and contribute to their inversion relative to each other, by mechanisms that are not currently well understood, in order to create an equimolar solution of four genomic isomers. Additionally, 75 known open reading frames code for proteins required in processes of nucleotide metabolism, genome replication, and viral proliferation. Finally, there are three origins of replication on the HSV-1 genome, one located in the U_L region – ori_L – and two flanking the U_S region - ori_S (Figure 1). Interestingly, replication can occur without the utilization of any of these origin sites by mechanisms that are still poorly understood (2, 3).

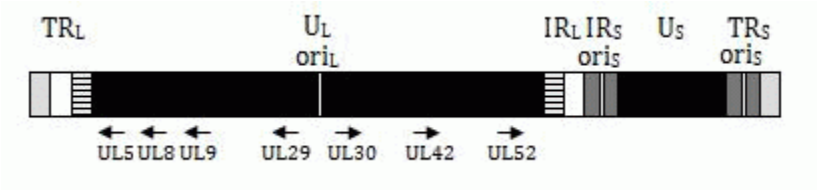

Figure 1: Diagram of the HSV-1 genome (not to scale); Unique long (U_L) region coding for seven essential early gene products (UL5, UL8, UL9, UL29, UL30, UL42, and UL52) with arrows representing direction of transcription; relative positions of three origins of replication (ori_L and two ori_S), unique short (U_S) region, terminal repeat (TR_L, TR_S) and internal repeat (IR_L, IR_S) sequences shown.

There are two ways in which viral DNA replication has been shown to occur in general. In the first model, daughter strands are synthesized by the formation of a bi-directional replication fork and initiation of replication via RNA primers in a semi-discontinuous manner. In the second model, daughter DNA strands are produced by strand displacement synthesis. HSV-1 DNA synthesis proceeds by the first method (2). However, in a linear genome without telomeres, loss of genetic information would occur at the ends of the chromosome because of limitations in the ability of the replisome to duplicate them. To mitigate this loss, HSV-1 undergoes a mechanism of requisite circularization after localizing to the nucleus and before commencing replication. Subsequently, HSV-1 initiates a process of sigma or rolling-circle replication to create long, head-to-tail concatemers. The creation of these "endless" strands consisting of multiple units of viral genome eliminates the need for a mechanism to duplicate the ends of the DNA strands, simply by allowing for the splicing of unit-length segments for packaging at the appropriate stage in replication. This rolling-circle hypothesis has been supported by the visualization of long, extensively

branched DNA structures, including forks, loops, and hairpins, in the nuclei of cells where HSV-1 replication is occurring (3).

Viral replication is a highly coordinated process, with defined temporal phases and enzymatic organization. HSV-1 immediate early (α) genes are expressed soon after genomic entry to the host nucleus when productive, lytic infection is ongoing, and function as transactivators of early (β) gene expression. The early gene products are created from seven genes in the U_L region – *UL5, UL8, UL9, UL29, UL30, UL42,* and *UL52* – and constitute the viral DNA replication machinery that is necessary for viral proliferation (3). Early gene products function as DNA polymerase (UL30/42) and helicase-primase (UL5/8/52) enzyme complexes, single-stranded DNA-binding protein (UL29), and an origin-binding protein (UL9). Their expression peaks between 5-7 hours post-infection and the synthesis of new DNA reaches a maximum 7-10 hours post-infection before the late (γ) capsid and structural genes are expressed, signaling the end of replication (3).

2.4 The HSV-1 Helicase-Primase Enzyme Complex

The UL5/8/52 helicase-primase complex consists of a heterotrimeric structure in which the UL5, UL8, and UL52 gene products are represented in a 1:1:1 ratio (9). This holoenzyme is associated with DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase, and DNA-dependent RNA primase activities. Together, the UL5/52 core subcomplex has been shown to retain DNA-dependent helicase, primase, and NTPase activities; the function of UL8 has not yet been well-classified (12-14). The UL5 subunit is a 97kDa protein that contains 5'-3' lagging-strand helicase activity and an NTP-binding motif

(17). The UL52 subunit is a 120kDa protein that is directly involved in the synthesis of RNA primers (15, 16). The activities of these enzymes are closely coordinated, though the exact nature of their interaction is still poorly understood (11, 12). Efforts to isolate the putative activities of the holoenzyme and attribute them to one subunit or the other have been complicated by the apparent interdependence between UL5 and UL52 which prevents the retention of relevant functions when the subunits are separated (13-17, 23).

UL5 likely functions as a DNA helicase and NTPase, unwinding double-stranded DNA at a rate of two nucleotides per second (9). The helicase-primase complex shows distinct 5'-3' translocation polarity, indicating a preference for the lagging strand in DNA synthesis (1). Sequence analysis of UL5 identifies seven motifs that are well-conserved in known and putative superfamily I DNA helicases. Two of these motifs define a nucleotide binding site, giving the enzyme NTPase function (17). Mutations in these motifs eliminate both helicase and NTPase activity, supporting the idea that UL5 acts as a lagging-strand helicase.

Similarly, the evidence regarding the function of UL52 is consistent with this subunit acting as a DNA primase (1). It has a divalent metal-binding DXD motif in its active site that is conserved in DNA polymerases and primases found in other viral, bacterial, and eukaryotic species. Mutations engineered in this and other subunit motifs conserved among DNA primases and polymerases have resulted in the elimination of primase activity without affecting helicase or NTPase activities, providing strong support for the involvement of UL52 in primer synthesis (15). UL52 produces oligoribonucleotides 6-13 base pairs long, presumably on both the leading and lagging strands of DNA. This has

complicated our understanding of UL5/52, as the functional directionality of each subunit is contradictory. There is much that remains to be understood about this subcomplex.

The additional UL8 subunit stimulates primer synthesis and interacts with UL30, UL42, ICP8 (product of *UL29*), and UL9 via its C-terminal region. This sort of C-terminal protein-protein interaction has been observed in other HSV-1 enzyme complexes using immunological coprecipitation assays and is proposed to constitute the mechanism by which replisome components are recruited to the replication origin (3, 18, 22). This suggests that one of the functions of UL8 is similar – that is, UL8 may help in the recruitment of the UL5/8/52 complex to the origin by interacting with proteins such as the origin-binding protein, UL9. Additionally, the UL8 subunit, when coordinated to the UL5/52 subcomplex to create the heterotrimeric UL5/8/52 helicase-primase, increases the rate of primer synthesis by approximately three-fold, without affecting K_M for DNA of UL5/52 alone (ca. 15μM nucleotides), or the rate of DNA-dependent ATPase and helicase activities (2). In the absence of UL8, primer elongation is largely abolished, indicating the necessity for this enzyme during viral replication *in vivo* (17). While the purpose of UL8 remains unclear, it is not, in any case, required for DNA-dependent NTPase or helicase activities (13).

2.5 The HSV-1 Polymerase Enzyme Complex

The UL30/42 heterodimer is composed of a 136kDa UL30 subunit and a 51kDa UL42 subunit (2). This complex is the target of numerous nucleoside analogue-derived drugs.

UL30 is the HSV-1 DNA polymerase. It shares considerable sequence similarity to other viral and cellular DNA polymerases, and is classified as a POL α polymerase (24). The enzyme uses gapped DNA, hairpin DNA and single-stranded oligodeoxyribonucleotides as substrates (2). In addition to polymerase activity, UL30 has intrinsic 3'-5' exonuclease activity which helps to ensure high fidelity of replication, as well as RNase H activity which allows it to remove RNA primers and replace them with deoxyribonucleosides (2). As stated previously, UL30 interacts with the C-terminal domain of UL8 to be recruited to the initiation complex at the start of replication. The recruitment of the polymerase complex to an initiation scaffold consisting of UL5/8/52, UL9, and ICP8 may require primer synthesis by a functional UL52 subunit (14, 15).

The 65kDa UL42 subunit of the enzyme complex is the HSV-1 DNA polymerase processivity factor (21). It is therefore a functional analogue of the *Escherichia coli* β clamp. However, unlike the *E. coli* β clamp which encircles DNA and requires a clamp-loading complex, HSV-1 UL42 binds double-stranded DNA directly and thus bypasses the necessity for a clamp-loader (21). The N-terminal domain of UL42 is the region which allows binding to duplex DNA and association with UL30. Conversely, the mechanistic association of UL30 to UL42 is mediated by the UL30 C-terminal domain. UL42 also enhances the activity of UL9, which, in addition to functioning as the origin-binding protein is thought to act as a leading-strand helicase (3).

The UL30/42 heterodimer binds to DNA with a K_A of 1×10^8 M^{-1}, and the UL42 processivity factor increases the affinity for DNA of UL30 by ten-fold. Additionally, UL42 increases the DNA-enzyme complex K_A by five-fold and decrease its K_D by thirty-fold. UL42 has no effect on the pre-steady-state rate constant for the incorporation of correct

nucleotides by UL30, but prevents misincorporation by reducing the rate constant for polymerization of mismatched bases by four-fold.

This study addresses replication of the viral genome, with specific emphasis on the mechanistic consequences of coupling between UL5/8/52 and UL30/42. We are interested specifically in the potential kinetic effects resulting from interactions between these enzymes. Ultimately, results of the study may help us better understand mechanisms involved in viral gene replication and lend to novel approaches in drug therapies. HSV-1, like many other viruses, serves as a simple model of basic DNA replication, about which much is still unknown.

3 MATERIALS AND METHODS

3.1 MATERIALS

Baculovirus-infected Sf9 cells had previously been obtained from the University of Colorado Health Sciences Center Tissue Culture Core Facility. These cells were either triply-infected with UL5, UL8, and UL52, or doubly-infected with UL30 and UL42. Cells were grown and harvested after 48 h of infection and protein was purified using nickel nitrilotriacetic acid chromatography as described previously (13).

Synthetic oligonucleotides of defined sequence were purchased from Integrated DNA Technologies, Inc. The sequences are depicted in Figure 2B. [α-^{32}P]dNTPs were purchased from PerkinElmer, Inc. All other reagents were of the highest available commercial quality.

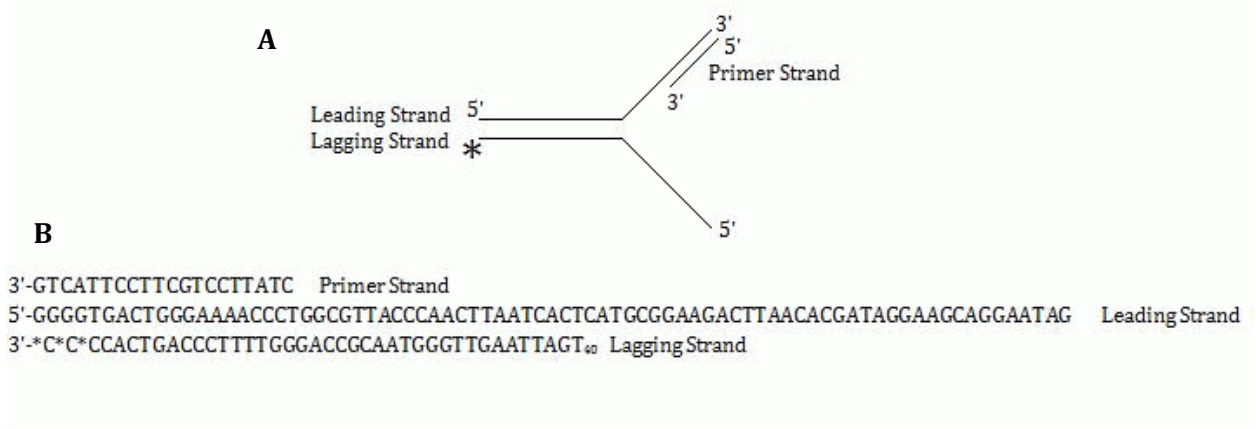

Figure 2: Schematic of template DNA used. *A* Radiolabeled leading strand/lagging strand forked DNA with DNA primer annealed at the leading-strand 3' end. *B* Complementary synthetic oligodeoxyribonucleotide sequences.

3.2 METHODS

3'-RADIOLABELING OF DNA TEMPLATES. Synthetic oligoribonucleotides were gel-purified and concentrations were determined using Nanopore. A two-stranded DNA fork was created by heating leading and lagging strands in a 1:1.25 molar ratio with 50 μM $MgCl_2$ at 95°C for 5 min and cooled to room temperature. The resulting 2.0 μM DNA fork was labeled at the 3' end of the lagging strand using 1 μCi [α-^{32}P]dCTP, Klenow fragment, and 1x New England Biolabs Buffer 2. After incubation at 37°C for 30 min, the solution was heated to 95°C for 15 min to fully denature Klenow and cooled to room temperature. Finally, the resulting 1.0 μM DNA radiolabeled fork was annealed to the primer strand by heating to 95°C for 5 min and cooled to room temperature.

HELICASE ASSAY. Reactions typically contained 20 mM HEPES, pH 7.6, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 10% glycerol, 1 mM $MgCl_2$, and 1 mM ATP. Assays included 45 μM of all four dNTPs when indicated. Reaction mixtures also contained 25 nM [α-^{32}P] radiolabeled fork/primer DNA template as well as 250 nM lagging strand trapping DNA to prevent reannealing to the leading strand after conversion by helicase-primase. Assays were initiated by adding 60 nM UL5/8/52, incubated at 37°C for up to 60 min, and quenched by adding gel-loading buffer consisting of 15 mM EDTA, 1% SDS, and 50% glycerol. Controls consisted of boiled DNA to obtain labeled single strand. Products were separated on 13% native polyacrylamide gel run at 4°C for 12 h at low voltage and visualized by phosphorimagery. Quantitative analysis was performed using ImageQuant software.

POLYMERASE ASSAY. Reactions typically contained 20 mM HEPES, pH 7.6, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 10% glycerol, 1 mM $MgCl_2$, 45 μM each dATP, dGTP, and dCTP, and 1 μCi [α-^{32}P]dTTP. Reaction mixtures also contained 25 nM unlabeled fork/primer DNA template. Assays were initiated by adding 5 nM UL30/42 exo(+), incubated at 37°C for up to 60 min, and quenched by adding gel-loading buffer consisting of 15 mM EDTA, 1% SDS, and 50% glycerol. Products were separated on 13% native polyacrylamide gel, run at room 4°C for 12 h at low voltage and visualized by phosphorimagery. Quantitative analysis was performed using ImageQuant software.

ASSAY TESTING HELICASE IN PRESENCE OF POLYMERASE. Reactions contained 20 mM HEPES, pH 7.6, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 10% glycerol, 1 mM $MgCl_2$, and 25 nM radiolabeled fork/primer DNA template. Additionally, reactions contained 1 mM ATP, 250 nM trapping DNA, and 45 μM of all four dNTPs when indicated. Assays included and were initiated by addition of both 60 nM UL5/8/52 and 5 nM UL30/42 exo (+), incubated at 37°C for up to 60 min, and quenched by addition of gel-loading buffer consisting of 15 mM EDTA, 1% SDS, and 50% glycerol. Labeled DNA was boiled at 95°C for 5 min to create a single-stranded control. Products were separated on 13% native polyacrylamide gel run at 4°C for 12 h at low voltage and visualized by phosphorimagery. Quantitative analysis was performed using ImageQuant software.

ASSAY TESTING POLYMERASE IN THE PRESENCE OF HELICASE. Reactions contained 20 mM HEPES, pH 7.6, 0.1 mg/mL bovine serum albumin, 1 mM dithiothreitol, 10% glycerol, 1 mM $MgCl_2$, and 25 nM unlabeled fork/primer DNA template. Additionally, reactions contained 50 mM ATP,

250 nM trapping DNA, 1 μCi [α-^{32}P]dTTP and 45 μM of all other dNTPs when indicated. Assays included and were initiated by addition of both 60 nM UL5/8/52 and 5 nM UL30/42 exo (+), incubated at 37°C for up to 60 min, and quenched by addition of gel-loading buffer consisting of 15 mM EDTA, 1% SDS, and 50% glycerol. Labeled DNA was boiled at 95°C for 5 min to create a single-stranded control. Products were separated on 13% native polyacrylamide gel run at 4°C for 12 h at low voltage and visualized by phosphorimagery. Quantitative analysis was performed using ImageQuant software.

4 RESULTS

Duplication of the HSV-1 genome takes place at a replication fork, in much the same way that eukaryotes and *E. coli* are known to replicate. It is necessary for the helicase-primase complex to track along the DNA towards the still-duplexed DNA at the fork and unwind it, leading the way for the polymerase complex to follow and incorporate nucleotides into the growing daughter strand (Figure 3). UL5/8/52 is relatively inefficient, by some reports unwinding only two nucleotides per second (9). Furthermore, even when enzyme concentration is in excess relative to DNA substrate, unwinding is not significantly enhanced. On the other hand, UL30/42 is estimated to polymerize dNTPs at a rate of 150 per second (2). Based on this knowledge, we hypothesized that interactions between the helicase-primase and polymerase enzymes may enhance the rate of helicase-primase, allowing for more rapid unwinding.

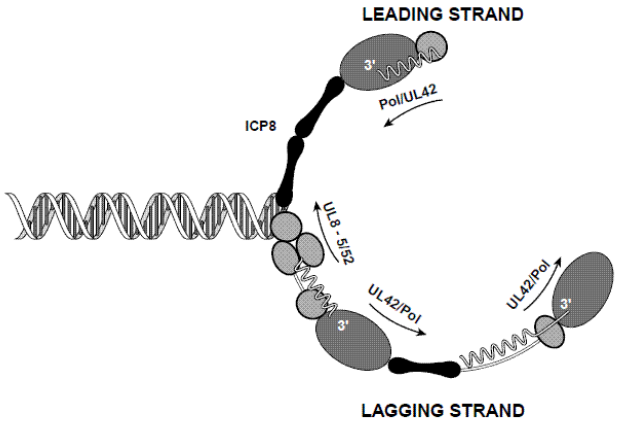

Figure 3: Illustration of the HSV-1 replication fork. UL5/8/52 can be seen moving along the lagging strand toward duplexed, helical DNA at the left of the figure; UL30/42, here called Pol/UL42 starts at RNA primers and polymerizes dNTPs into the forming daughter strands 5'-3', following UL5/8/52 on the leading strand. Image from Boehmer, P.E. and Lehman, I.R. (1997) *Herpes Simplex Virus DNA Replication*. Annu. Rev. Biochem. 66, 347-384.

PRESENCE OF HSV-1 UL30/42 DOES NOT ENHANCE UNWINDING BY UL5/8/52

To test the kinetic effects of adding UL30/42 exo (+) to UL5/8/52, we began by performing an experiment comparing helicase-primase activity on the fork/primer DNA substrate shown in Figure 2 with an assay containing both UL5/8/52 helicase-primase and UL30/42 exo (+) polymerase. UL5/8/52 can unwind labeled fork DNA lacking a primer annealed to the leading strand (2). Prior to beginning studies using the fork/primer template (Figure 2), we determined that the addition of a DNA primer did not affect activity of the helicase (data not shown).

Each of the following assays was incubated at 37°C and quenched at time points of 1 min, 15 min, 30 min, and 60 min; products were separated by non-denaturing PAGE (Figure 4). Helicase-primase activity was present under both conditions, as indicated by increased visualization of single-stranded DNA products over time. Previously, it had been shown that helicase-primase was able to unwind substrates with a single-stranded 5' overhang, including simple forked DNA without attached primer and a three-way junction substrate (1). To our knowledge, this is the first experiment testing UL5/8/52 activity using forked DNA with a primer bound to the leading strand.

There is no significant quantitative effect of UL30/42 exo (+) on unwinding of the substrate by UL5/8/52; the rates of unwinding are largely the same both with and without UL30/42 exo (+) (Figure 5). The lack of rate enhancement in helicase activity shown by this comparison of UL5/8/52 activity alone versus UL5/8/52 with UL30/42 exo (+) present is unexpected. There are, however, many variables that may contribute to this result.

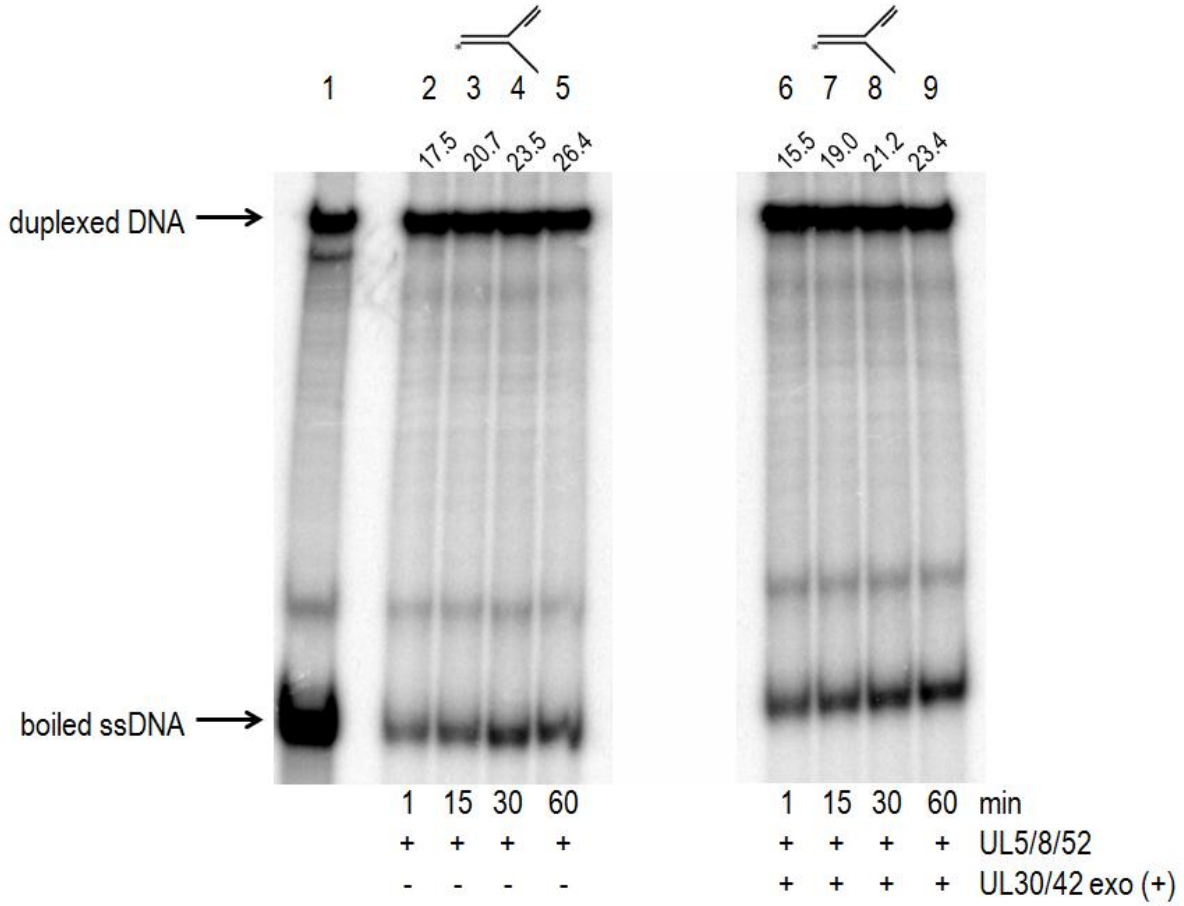

Figure 4: Effect of UL30/42 exo (+) on helicase-primase activity of UL5/8/52. 13% native PAGE depicting results of helicase-primase assay. Lanes 2-5 show results of helicase-only assay with increasing time points from left to right. Lanes 6-9 show results of helicase assay in conjunction with UL30/42 exo (+). There is an increase in the percentage of unwound DNA over time. Boiled DNA control in lane 1 indicates the position of converted DNA.

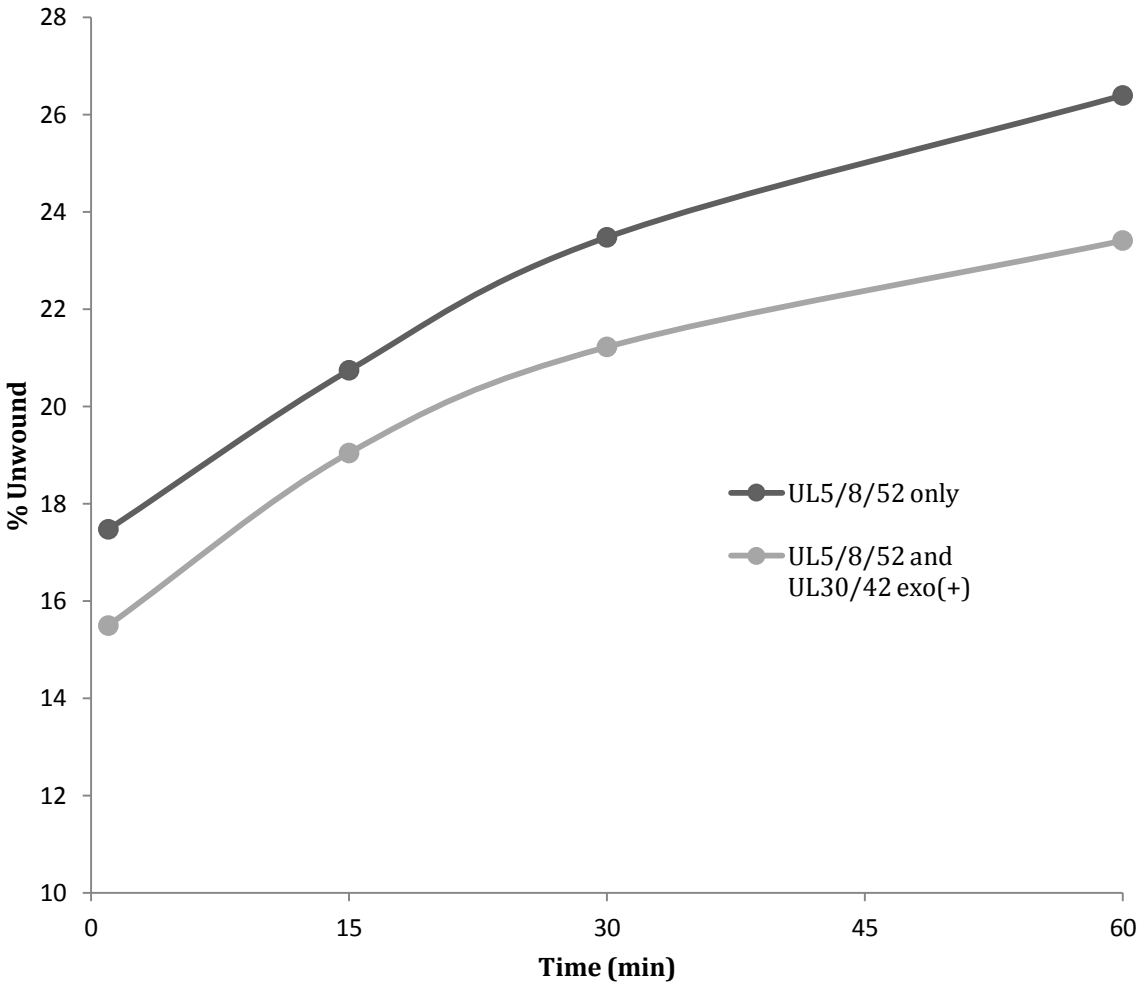

Figure 5: Kinetics of unwinding by UL5/8/52 both in the presence and absence of UL30/42 exo (+) over time. Light grey represents unwinding in the presence of UL30/42 exo (+); helicase activity by UL5/8/52 (light grey) is diminished in these conditions as compared to UL5/8/52 without UL30/42 exo (+) (dark grey).

EFFECT OF NUCLEOTIDE SUBSTRATES ON ABILITY OF UL/8/52 TO UNWIND DNA

To further demonstrate the effects of UL30/42 exo (+) on the rate of double-stranded DNA unwinding by UL5/8/52 at a replication fork, we compared the activity of UL5/8/52 under several different conditions. Assays were visualized using native PAGE (Figure 6). Upon quantification of single-stranded DNA formed by UL5/8/52, we found that presence of ATP and dNTPs in solution affected the rate of conversion of fork/primer DNA to single-stranded DNA.

Figure 6 shows the results of six assays under varying conditions. Lanes 2-5 (Assay 6A) show unwinding of radiolabeled fork/primer DNA in the presence of 60 nM UL5/8/52, 5 nM UL30/42 exo (+), 1 mM ATP, and 45 μM dNTPs at time points of 0 min, 15 min, 30 min, and 60 min. Unwinding occurs, with the percentage of unwound substrate increasing over time. While the amount of single-stranded DNA produced appears unimpressive, this inefficient unwinding is consistent with data reported previously in other studies (1). Lanes 10-13 (Assay 6C) show conversion of substrate to single-stranded DNA in the absence of UL30/42 exo (+), with dNTPs still present. Formation of single-stranded DNA is apparent in this set of data as well, increasing with time. Lanes 14-17 (Assay 6D) show unwinding of double-stranded DNA in the absence of both UL30/42 exo (+) and dNTPs. Like Assays 6A and 6C, Assay 6D demonstrates formation of single-stranded DNA, with the percentage unwound increasing over time.

Quantification of single-stranded DNA formed showed that the presence of dNTPs and absence of UL30/42 exo (+) in the reaction mixture resulted in the inhibition of helicase activity. The rate of unwinding during the initial 15 minutes of UL5/8/52 in the absence of both UL30/42 exo (+) and dNTPs (Assay 6D) is somewhat more rapid than that

of UL 5/8/52 in the presence of both (Figure 7). However, the maximum percentage unwound at 60 min by UL5/8/52 under each of these conditions is almost equivalent. 16% of DNA was unwound in Assay 6A, while Assay 6D displayed 16.5% unwinding of total DNA. Meanwhile, DNA unwound by UL5/8/52 when dNTPs were present in the reaction without UL30/42 exo (+) was diminished. UL5/8/52 activity in Assay 6C was significantly slower, ultimately only unwinding 11.5%, and did not recover at any point within the 60 min tested. Thus the presence of excess dNTPs inhibits unwinding of DNA duplex by UL5/8/52. dNTPs may act as competitive inhibitors of the ATP and GTP-binding sites of UL5/8/52. Inability of UL5/8/52 to obtain useable energy from dNTP hydrolysis would hinder melting of duplex DNA.

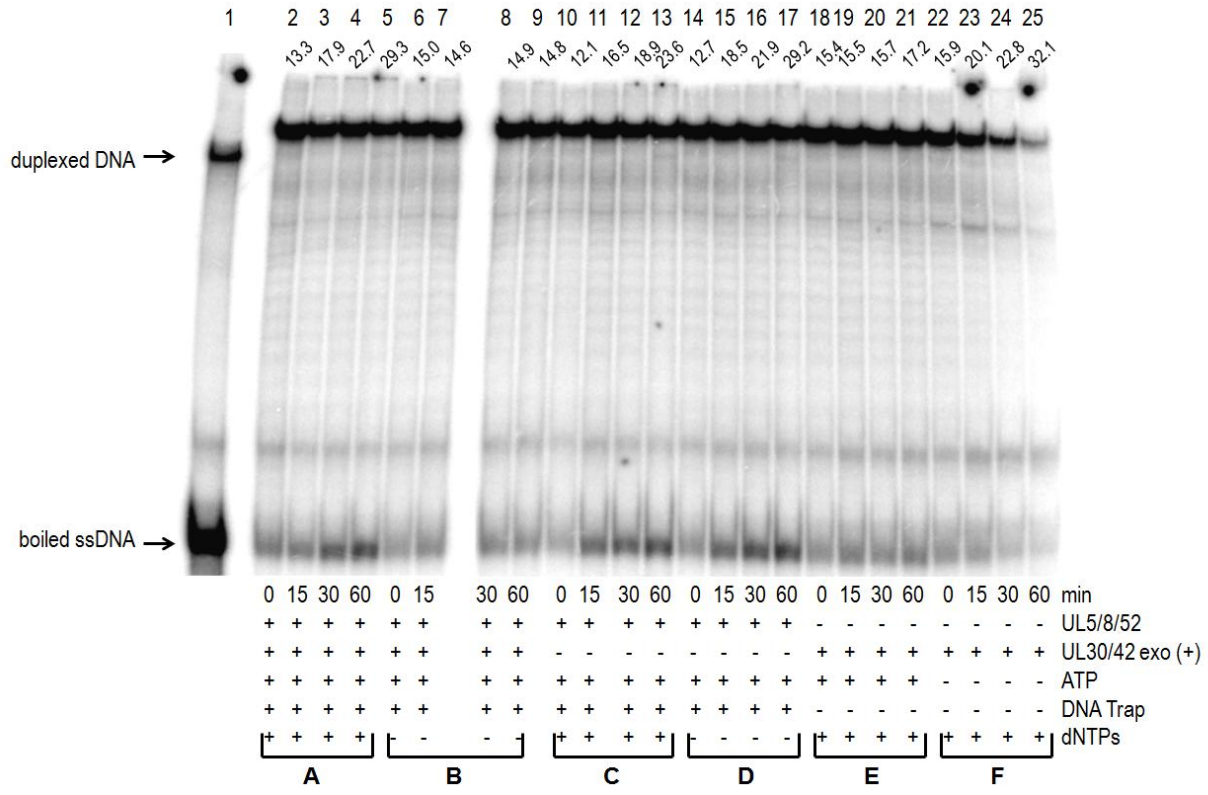

Figure 6: Comparison of six assays exploring the effects of ATP, dNTPs, and UL30/42 exo(+) on helicase activity of UL5/8/52. Fork/primer DNA substrate (see Materials and Methods, Figure 2) was used in all assays. Trapping DNA was included in all assays including helicase (A, B, C, and D) and omitted in assays involving only polymerase (E and F). Lanes 2-5 show Assay A, containing all reagents; respectable levels of unwinding was observed. Lanes 6-9 show Assay B which investigated the effects on UL5/8/52 of omitting dNTPs from the reaction mixture; no appreciable unwinding was observed. Lanes 10-13 show the results of Assay C, which depicts the effect on UL5/8/52 of dNTPs without UL30/42 exo (+); some unwinding of fork/primer DNA to single-stranded DNA is observed. Lanes 14-17 show Assay D, which contains UL5/8/52 without UL30/42 exo (+) and dNTPs; appreciable levels of unwinding are observed. Lanes 18-21 show data collected from Assay E, which contained UL30/42 exo (+), dNTPs, and ATP, but did not contain UL5/8/52. Assay E investigates the activity of UL30/42 exo (+) and any effect ATP may have had on the polymerase. Lanes 22-25 show Assay F which contained UL30/42 exo (+) and dNTPs in the absence of UL5/8/52 and ATP. Assay F was also used to investigate the activity of UL30/42 exo (+).

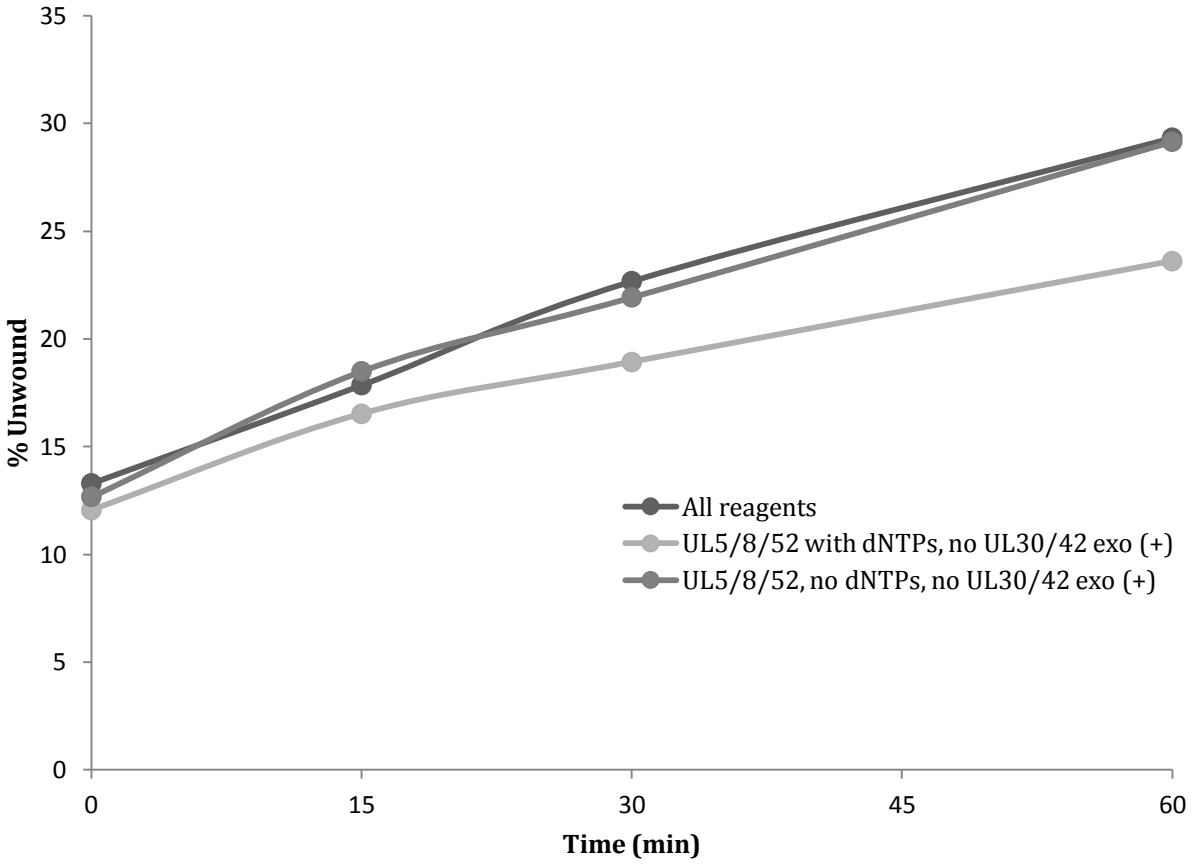

Figure 7: Comparison of % DNA unwound by UL5/8/52. Assay 6A contained all reagents including UL5/8/52, UL30/42 exo (+), dNTPs, and ATP. Assay 6C did not contain UL30/42 exo (+); Assay 6D did not contain UL30/42 exo (+) or dNTPs. It can be seen that Assay 6D initially displayed a higher rate of unwinding than Assays 6A and 6C. Assay 6C maintained a slower rate of unwinding throughout the 60 min assay, while 6A and 6D ultimately resulted in comparable percentages of unwound DNA.

EFFECTS OF VARYING CONCENTRATION OF UL30/42 EXO (+)

We also investigated the effect on unwinding of UL30/42 exo (+) in the absence of dNTPs. Lanes 3-9 (Assay 6B) depict unwinding of fork/primer DNA by 60 nM UL5/8/52 in the presence of 5 nM UL30/42 exo (+), 1 mM ATP, and no dNTPs. Visualization by phosphorimagery showed a distinct absence of helicase activity; single-stranded DNA is not seen. Indeed, quantification confirmed that single-stranded DNA was not formed by UL5/8/52 (Figure 8).

The same assay (Assay 6B) was performed with 2 nM UL30/42 exo (+). At this lower concentration of polymerase, 27.5% of DNA was single-stranded at 60 min with total unwinding of 20.7%. Figure 8 compares the progress of these reactions. There is significantly more unwinding by UL5/8/52 with 2 nM UL30/42 exo (+) than with 5 nM UL30/42 exo (+). This indicates that UL30/42 exo (+) itself is somehow hindering UL5/8/52, which is unexpected and counterintuitive. However, this can be explained if UL30/42 exo (+) can hydrolyze ATP when dNTPs are missing, causing it to compete directly with UL5/8/52 for nucleotide substrate. While UL30/42 polymerase does not require ATP as an energy source, it is a necessary substrate for UL5/8/52, which uses the energy released from NTP hydrolysis to melt apart DNA base pairs.

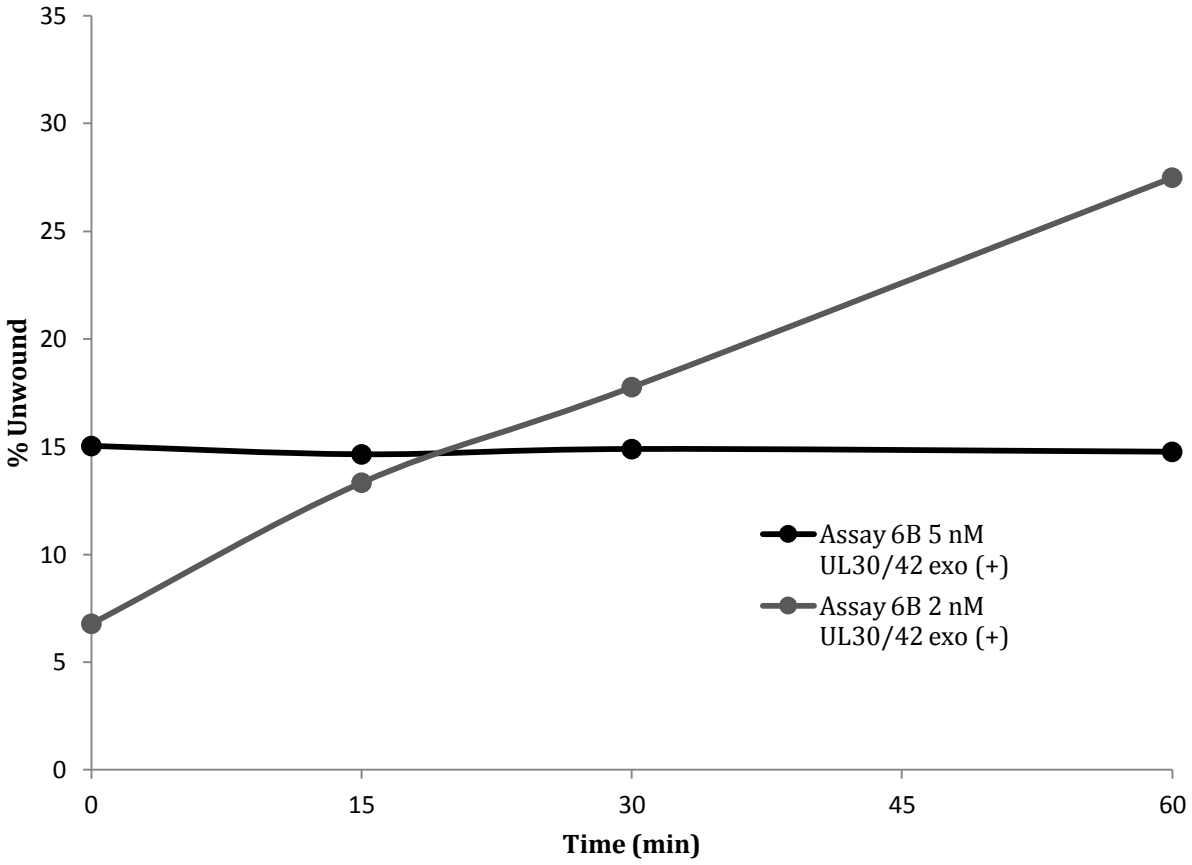

Figure 8: Graph comparing effects of UL30/42 exo (+) concentration. Assay 6B included UL5/8/52, UL30/42 exo (+), and ATP, but did not contain dNTPs. It can be seen that the rate of unwinding is dependent in part upon the concentration of UL30/42 exo (+) present in solution. At 5 nM UL30/42 exo (+), the percentage of single-stranded DNA does not appear to increase. At 2 nM UL30/42 exo (+), there appears to be significant unwinding

	UL5/8/52 and 5 nM UL30/42 exo (+)				UL5/8/52 and 2 nM UL30/42 exo (+)			
Time (min)	0	15	30	60	0	15	30	60
% Unwound	15.0	14.6	14.9	14.8	6.8	13.3	17.8	27.5

Table 1: Numerical representation of percent unwound DNA shown in Figure 8.

UL30/42 ACTIVITY IN INHIBITION OF UL5/8/52

The previous data show that UL30/42 exo (+) may have unexpected effects on the process of DNA unwinding by UL5/8/52. To investigate what these effects may be, assays were conducted in which only UL30/42 exo (+) activities were examined. We found that UL30/42 exo (+) may be exhibiting phosphatase activity in addition to polymerization of dNTPs, which interfered with accurate quantification of helicase activity, and made it appear upon initial analysis as though ATP is influencing experimental outcomes.

Figure 6 lanes 18-21 (Assay 6E) and lanes 22-25 (Assay 6F) together show the results obtained from reaction conditions which tested the effects, if any, of ATP and dNTPs on UL30/42 exo (+) function. Assay 6E contained ATP, while Assay 6F did not. The relative levels of single-stranded DNA in Assay 6E stayed constant throughout the 60 minutes (Figure 9A). In contrast, relative levels of single-stranded DNA measured in Assay 6F increased by 16.2%, which is comparable to that observed in Assay 6D (16.5%), in which UL5/8/52 activity was measured alone (Figure 9B). One interpretation of these results is that ATP directly interacts with and affects the polymerase. However, there is a decrease in the total amount of radiolabeled DNA over time (Assay 6F), indicative of phosphatase activity clipping off the triphosphate groups of [α-^{32}P]dCTP used in the labeling process, reducing detectable signal during phosphorimagery. This would make it appear as though the relative percentage of single-stranded DNA is increasing.

Polymerase activity was further explored by performing the same set of six assays depicted in Figure 6, but with the substitution of unlabeled fork/primer DNA and [α-^{32}P]dTTP for radiolabeled DNA substrate and cold dTTP (Figure 10). In the presence of ATP, incorporation of dNTPs progresses in much the same fashion in both Assays 10E and

10A. Incorporation of UL30/42 exo (+) can also be visualized in Assay 10F. However, the monotonic increases of dNTP incorporation over time that occur in Assays 10A and 10E are not seen in Assay 10F.

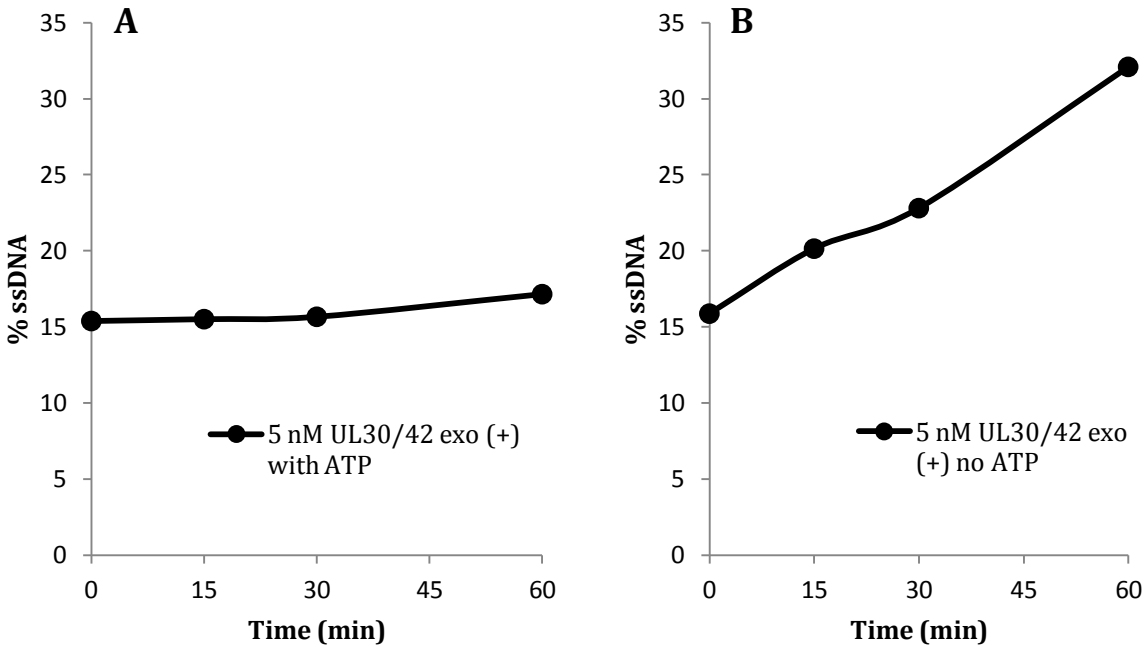

Figure 9: Graphs comparing ATP effects on UL30/42 exo (+) activity. Assay 6E contained both 5 nM UL30/42 exo (+) and ATP, along with dNTPs. Assay 6F contained only 5 nM UL30/42 exo (+) and dNTPs, but did not include ATP. The relative percentage of single-stranded DNA shows significant increase in Assay 6F, but did not increase substantially in Assay 6E.

The conditions of Assay 10A support a steady increase in dNTP incorporation, with a maximum of 53.7% of DNA showing incorporation by 60 min (Figure 11). However, the amount of DNA showing incorporation never reaches a constant percentage, likely resulting from unwinding by helicase allowing more DNA to be replicated. Assay 10E also initially shows a steady increase in dNTP incorporation; but the rate of this initial phase is

much more rapid than the rate of increase in Assay 10A (Figure 11). Assay 10E reaches a constant percentage of dNTP incorporation, indicating that strand-displacing synthesis is not occurring and that UL30/42 exo (+) has not run out of dNTPs to hydrolyze, allowing for steady-state equilibrium to be achieved. In contrast, Assay 10F shows distinct reaction progression. In the first 15 minutes of the reaction, incorporation of dNTPs reaches a maximum level of 36.8%. Subsequently, the amount of incorporation decreases and then levels out to 29%. The anomalous data point at 15 min may be the result of a loading error, in which case the progression of the reactions is likely to follow a similar path, indicating that ATP does not significantly affect UL30/42 exo (+) polymerase activity. The absence of ATP may affect total incorporation, but the data collected are far from conclusive. Also, as this is a native gel, individual products cannot be distinguished. Thus we cannot conclude that ATP directly affected these results. However, it is clear that UL30/42 exo (+) is functional in these assays. This is important in the analysis of UL5/8/52 helicase activity.

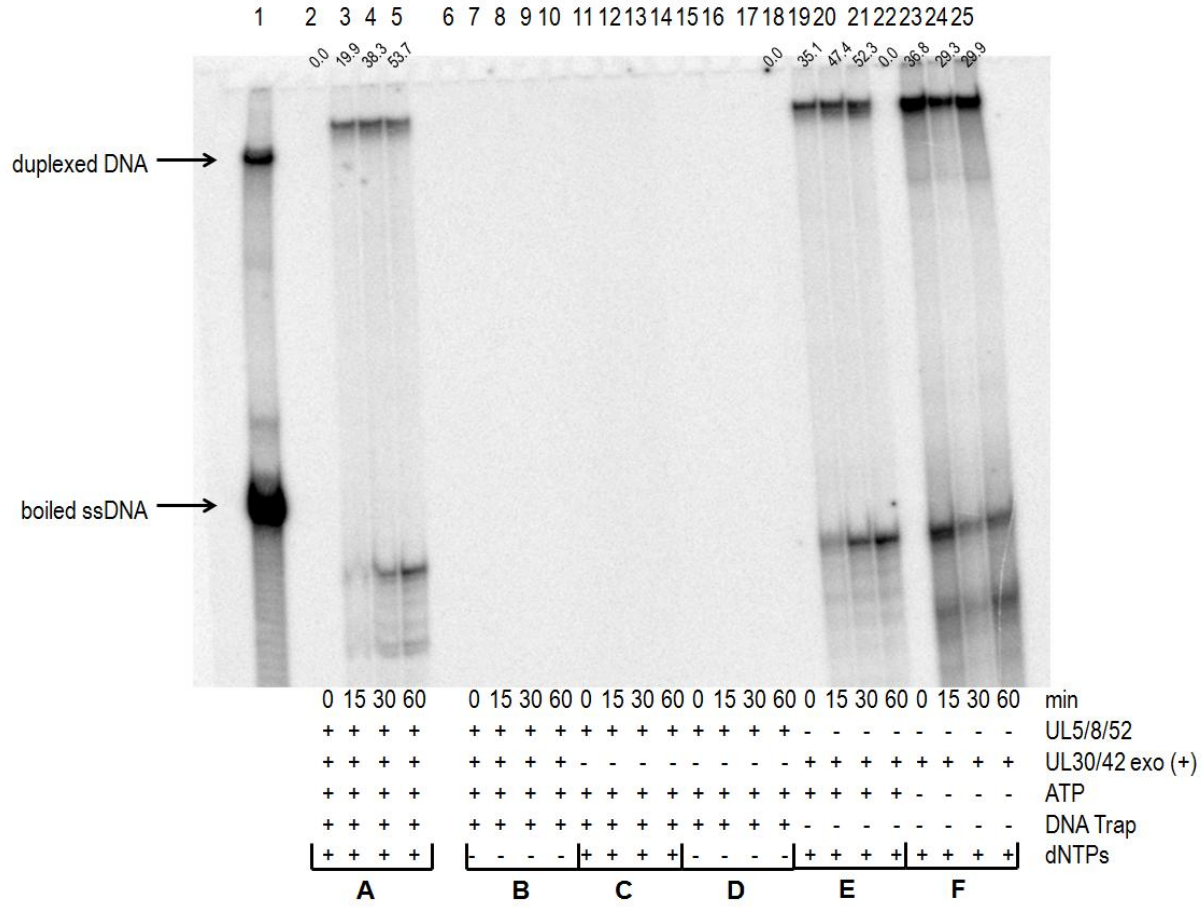

Figure 10: Comparison of six assays exploring the effects of ATP, dNTPs, and UL30/42 exo(+) on helicase activity of UL5/8/52. Unlabeled fork/primer DNA substrate was used in all assays. Trapping DNA was included in helicase assays and omitted in assays involving only polymerase. Lanes 2-5 show Assay A, containing all reagents; incorporation of [α^{32}-P]dTTP can be observed. Assays B, C, and D (lanes 6-9, 10-13, and 14-17) omitted either dNTPs or UL30/42 exo (+) from the reaction mixture; as expected, no incorporation was observed. Lanes 18-21 show data collected from Assay E, which contained UL30/42 exo (+), dNTPs, and ATP, but did not contain UL5/8/52. UL30/42 exo (+) is clearly actively incorporating dNTPs. Lanes 22-25 show Assay F which contained UL30/42 exo (+) and dNTPs in the absence of UL5/8/52 and ATP. Assay F also displays clear UL30/42 exo (+) activity.

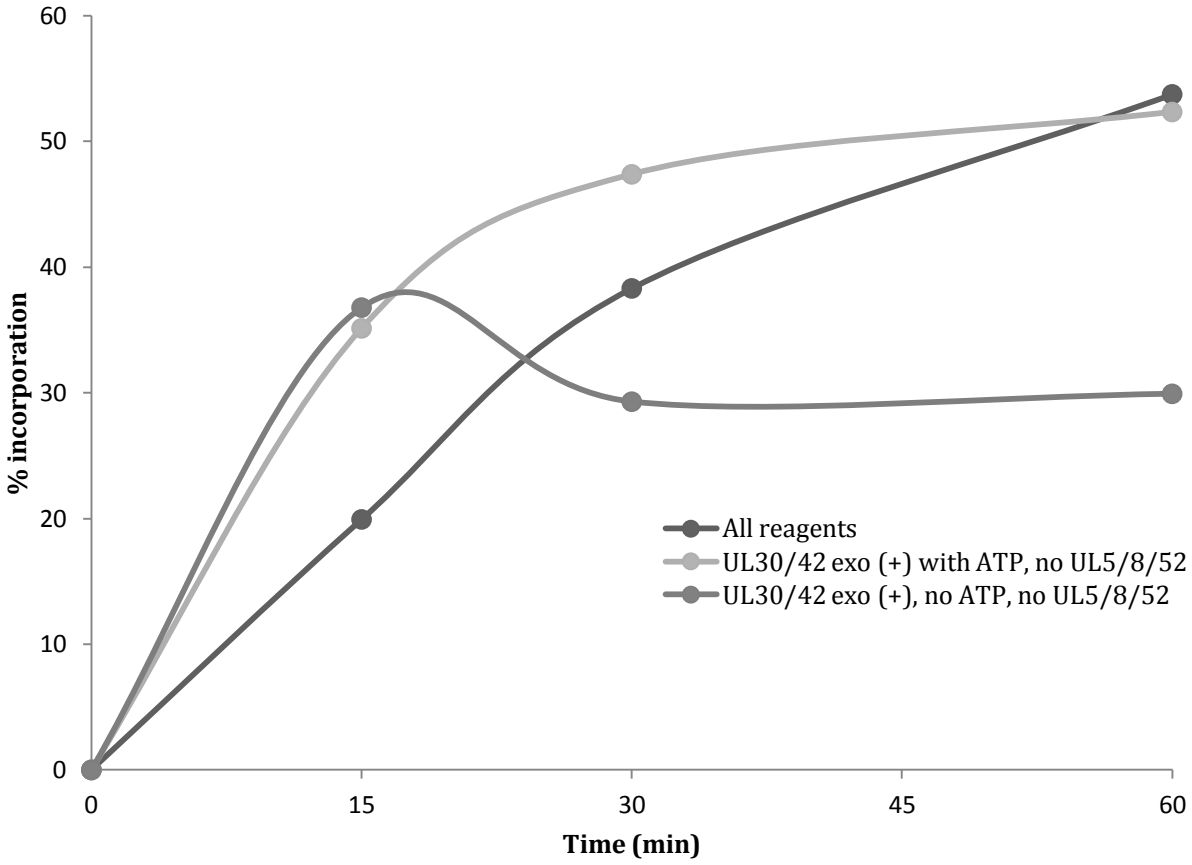

Figure 11: Graph comparing UL30/42 exo (+) activity. Assay 10A is represented in dark grey; incorporation was observed, the continuing increase in slope may indicate unwinding and exposure of more DNA to be replicated. Assay 10E is represented in light grey; incorporation is observed, the rate appears to be more rapid initially and reach steady-state. Assay 10F is represented in medium grey; after peaking, percent of DNA displaying dNTP incorporation appears to decrease before leveling out.

5 Discussion and Conclusions

The results obtained in this study were unexpected and leave many questions unanswered regarding the mechanisms of HSV-1 genome replication. The synergy hypothesized to exist between UL5/8/52 and UL30/42 exo (+) was not detected in any of the assays conducted through the course of the study. This may be the cause of a number of factors, including the low ratio of UL30/42 exo (+) to UL5/8/52, inherent inefficiency of the UL5/8/52 enzyme complex itself, variability in the reagents used as compared to other studies, and experimenter error.

Assuming that HSV-1 replicates in a manner involving unwinding and polymerization at replication forks semi-discontinuously, it should follow that UL5/8/52 as the HSV-1 helicase-primase moves in front of UL30/42 to melt apart base pairs so that they can be read and replicated. It is understood that UL5/8/52 is relatively inefficient, with a turnover rate much slower than that of UL30/42, leading to the main prediction of this study that these holoenzymes must act synergistically in order to achieve a more rapid combined rate of reaction. However, under none of the conditions studied was an increased rate detected when both enzymes were present in a single reaction.

The assays described within this study used a ratio of UL30/42 exo (+) to UL5/8/52 of either 1:12 or 1:30. Experiments in which the concentrations of each enzyme complex were equivalent have not been performed, due to time constraints. Though the ratio of UL30/42 exo (+) to UL5/8/52 may seem small, however, it has been hypothesized that UL5/8/52 may be capable of forming oligomeric complexes utilizing multiple UL5/8/52 heterotrimers, and that this may even be necessary to resolve the conundrum regarding

the contrary directionalities of helicase and primase function. Appropriate ratios of UL30/42 to UL5/8/52 may therefore be more difficult to determine. It is probable that the relative concentrations of UL5/8/52 to UL30/42 exo (+) may have significant consequences on the rate of unwinding detected for UL5/8/52. However, when UL30/42 exo (+) concentration was decreased by a factor of 2.5 as shown in Figure 8, total unwinding increased by about 21%, which is counterintuitive. Experiments further investigating the effects on the ratio of UL5/8/52 to UL30/42 exo (+) are necessary in order to show more definitive trends in this phenomenon.

Additionally, UL5/8/52 has been shown on several previous occasions to be a truly inefficient enzyme (1, 9, 16). In at least one previous study, UL5/8/52 was not shown to unwind more than approximately 35-40% of DNA substrate (16). This characteristic inefficiency is also evident in the data collected for this study. In the majority of experiments performed, UL5/8/52 is generally shown to unwind a maximum of approximately 30% of duplexed DNA under any of the conditions tested. Furthermore, many months were spent in initial assays optimizing the conditions of the reaction buffer finally used in the assays depicted here. The reasons for this inefficiency continue to be unclear.

UL5/8/52 has previously been shown to hydrolyze dNTPs (27). It is possible that the presence of dNTPs in solution inhibits helicase activity by competing with ATP for the UL5/8/52 NTPase active site. However, while it is shown in Figure 7 that UL5/8/52 activity diminishes when dNTPs are in solution as compared to activity when dNTPs are absent, the reason for this is not entirely clear. Perhaps hydrolysis of dNTPs does not supply UL5/8/52 with the energy needed to melt apart DNA base pairs in the same fashion

as ATP. While this may occur, it seems that hydrolysis of dNTPs by UL5/8/52 would influence the kinetics of UL30/42 exo (+) causing a marked decrease in the level of dNTP incorporation. However, there does not appear to be conclusive evidence to this effect.

It is also possible that the conditions used in these assays simply did not allow for optimal function by either UL30/42 or UL5/8/52. Though helicase assays were performed under conditions similar to those described in previous studies, subtle variations in experimental technique, quality of reagents, and buffer composition may have translated into profound effects on results. Due to time constraints, each of the assays described in this thesis were only able to be performed once. Duplication of these and development of further assays are necessary to determine the precise nature of what effects various experimental factors may have on helicase and polymerase activities.

The observations collected in this study may also represent some of the peculiar phenomena that lead to latency of HSV-1. Viruses are obligate parasites that must invade host cells in order to replicate and survive. However, their survival is at least partly dependent on evading detection by the host immune system. Human immune defenses against viral infection rely on the intracellular detection, degradation, and surface presentation of virus-produced particles by MHC-I to cytotoxic lymphocytes. The inefficient levels of genome duplication processes presented in this study may be representative of a larger phenomenon in which HSV-1 inhibits its own replication in order to avoid detection by the host. Viral latency is still poorly understood. Thus we conclude that the complex interaction between HSV-1 replication enzymes UL5/8/52 and UL30/42 exo (+) may be affected by the stoichiometry of the enzyme complexes and that further studies examining this interplay are warranted.

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